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(7)

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Datum/Date 16.10.95

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°.//Patent Nr./Patent No./Brevet n°.
IMA/FP5071907	82903424.8-2110/0092574

Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire
MOLECULAR BIOSYSTEMS, INC.

DECISION REVOKING THE EUROPEAN PATENT (ARTICLE 102(1) EPC)

The Opposition Division - at the oral proceedings dated ...21.9.95.
- has decided:

European Patent No. 0092574 is revoked.

[ ] Additional decision:

The reasons for the decision are enclosed.

POSSIBILITY OF APPEAL:
An APPEAL may be filed against this decision within TWO MONTHS after notification of the decision. The appeal is subject to the payment of a fee for appeal, laid down by the Rules Relating to Fees; your attention is drawn to Articles 106 - 108 EPC, the wording of which is enclosed.

OPPOSITION DIVISION:

FRIEBEL F K W Chairman	MOONEN P 1st Examiner
GOHLKE P M 2nd Examiner	Legally qualified examiner

Enclosures: Reasons for the decision (Form 2916, 11. pages) + Annex 1/2
Wording of Articles 106-108 EPC (Form 2019)
[X] Minutes of oral proceedings

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Datum/Date 16.10.95

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°.//Patent Nr./Patent No./Brevet n°.
24-1 OPPO 02	82903424.8-2110/0092574

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EPO Form 2331 09.94 7053342 to EPO postal service: 11/10/95

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Datum/Date 16. 10. 95

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°.//Patent Nr./Patent No./Brevet n°.
SDR/GWS/I.8409 OPPO 03	82903424.8-2110/0092574

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EPO Form 2331 09.94 7053343 to EPO postal service: 11/10/95

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Datum/Date
16. 10. 95

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°.//Patent Nr./Patent No./Brevet n°.
DR. BUDDE OPPO 04	82903424.8-2110/0092574

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Datum/Date 16.10.95

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°.//Patent Nr./Patent No./Brevet n°.
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EPO Form 2331 09.94 7053345 to EPO postal service: 11/10/95

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Datum/Date
16. 10. 95

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°.//Patent Nr./Patent No./Brevet n°.
4-E 2971 OPPO 06	82903424.8-2110/0092574

Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire
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EPO Form 2331 09.94 7053346 to EPO postal service: 11/10/95

82903424.8 REVO

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Datum/Date
16. 10. 95

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°.//Patent Nr./Patent No./Brevet n°.
58 530 M/FG OPPO 07	82903424.8-2110/0092574

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EPO Form 2331 09.94 7053347 to EPO postal service: 11/10/95

82903424.8 REVO

REVOCATION OF THE PATENT

according to Art. 102(1) EPC

Facts and Submissions

I.

European Patent number 0 092 574 was granted in response to European Patent Application number: 82903424.8, date of filing 08.10.82.
Claimed priority: 23.10.81 (US 314124).
The mention of the grant of the patent was published in European Patent Bulletin 92/18 of 29.04.92.

The Proprietor of the patent is MOLECULAR BIOSYSTEMS, INC. (represented by Mr. Armitage).

Notice of Oppositions were filed by the following Opponents:

I. On 08.01.93 by the Opponent HYBRIDON, INC. (represented by Dr. Volker Vossius). The Opposition was withdrawn with letter of 25.11.94.

II. On 26.01.93 by the Opponent Chugai Seiyaku Kabushiki Kaisha, Tokyo (represented by Dr. Volker Vossius).

III. On 27.01.93 by the Opponent APPLIED BIOSYSTEMS, INC. (represented by Mr. Ritter).

IV. On 29.01.93 by the Opponent Hoechst AG (represented by Dr. Budde).

V. On 29.01.93 by the Opponent GEN-PROBE INC. (represented by Mr. Moon).

VI. On 30.01.93 by the Opponent CIBA-GEIGY AG (represented by Dr. Maschio).

VII. On 29.01.93 by the Opponent Genta INC. (represented by Dr. Helga Kolb).

The two claims under consideration in the form as granted for all designated Contracting States except AT read as follows:

Claim 1: Therapeutic agent for selectively blocking the translation of m-RNA into a targeted protein, comprising a stabilized oligonucleotide of 14 to 23 bases having a base sequence substantially complementary to a portion of the coding region of the mRNA coding for said targeted protein.

Claim 2: Therapeutic agent according to claim 1, characterized in that the oligonucleotide is in a phosphotriester form.

In the course of the proceedings, the Proprietor provided a list, summarizing the cited documents by the different Parties, which is appended to this decision as annex I. At the start of the Oral Proceedings the Opposition Division provided the Parties with a supplementary list of the additionally filed documents, which is attached to this decision as annex II.

The relevant documents cited in this decision are in particular the documents published after the filing of the european patent application like D15, while also the prior art documents D2 and D7/D8 have been taken into consideration.

II.

All Opponents request the contested patent to be revoked in its entirety in accordance with Articles 99 and 100(b) EPC for lack of sufficiency of disclosure (Article 83 EPC), while in accordance with Article 100(a) EPC the Opponents request revocation on the grounds of lack of novelty (Article 54) and/or inventive step (Article 56).

Essentially, the Opponents put forward the following facts and arguments:

- With respect to the lack of novelty, it is submitted by Opponent III (Notice of Opposition) and Opponent VI (see letter of 18.08.95) that claims 1 and 2 are anticipated by the disclosures of documents D7 and D33.
- With respect to the lack of involvement of an inventive step, it is essentially submitted by the Opponents that the subject-matter of all claims is obvious to the skilled person on the basis of the combination of the teachings of documents like D2 and D7/D8.
- With respect to the lack of sufficiency of disclosure, it is essentially submitted by the Opponents that, taking into account Technical Decisions T 409/91 and T435/91, the guidance to the skilled person, provided by the specification of the patent, is not sufficient to carry out the invention, at least not without undue experimentation and not within the whole area that is claimed. It was submitted that no experimental data in detail have been provided in the specification, contrary to Article 83 in conjunction with Rule 27(1)(e). The later filed technical information was indicated to be not acceptable, as not supporting the examples of the

description and not demonstrating that the solution to the identified technical problem has been solved.

It was furthermore noted that in vivo, therapeutic activity should at least be directed to and demonstrated for a specific disease and that in fact even 10 years after the filing of the application no effective therapeutical use of antisense drug was known (see ref D44, page 347).

III.

Conversely, the Patentee requests rejection of the Opposition and to maintain the patent unamended.
In support of his request, he submits the following arguments with respect to the requirements of Article 83 EPC: The claimed subject-matter is sufficiently disclosed as the principle of the methods for finding the agents of claims 1 and 2 have been laid down to the skilled person in sufficient detail. The finding of these agents for use in a therapy needed only the identification of the target gene and further optimizing of the oligonucleotide with respect to for example length and stabilization without the application of inventive skill; many choices for optimizing were available at the priority date. The patent provides two examples indicating how the skilled person can proceed, and further experimental evidence was filed during examination.
Furthermore, the Opponents have failed to demonstrate e.g. by tests that the oligonucleotides referred to in the specification of the patent in suit or additionally filed are examples not fit to demonstrate the effect as claimed by the invention. Many patents claim

therapeutical compounds which do not end up in the market, because for some reason the clinical trials are detrimental. In the present invention the fundamental principle is claimed, which has later been demonstrated to be succesfull in obtaining the desired effect (e.g. see D15). The agents claimed in the patent are clearly limited to oligonucleotides succesfull in therapy.

IV.

All Parties have requested Oral Proceedings; these were held on 21.09.95: Opponent III was not represented during the Proceedings. The minutes of the Proceedings are in the file. Essentially, the arguments (limited to the requirements of Article 83 EPC only) delivered by the Parties in the Proceedings expanded on the earlier raised arguments.
No Official Action concerning substantive examination resulting in a preliminary opinion preceded the Oral Proceedings.

Reasons for the Decision

1.

The Oppositions are admissible, because they meet all the requirements of Articles 99(1) and 100 EPC and of Rules 1(1) and 55 EPC.

2. Sufficiency of Disclosure

i. The contested patent is revoked on the ground that the invention is not disclosed in a manner sufficiently

clear and complete for it to be carried out by a person skilled in the art (Art. 100(b) EPC).

Said person does not know at the priority date which instructions to follow to carry out the invention in a reliable way: the specification has not shown at least one example which instructs the skilled person and demonstrates that a therapeutic agent according to claim 1 is obtainable.

ii. The Proprietor has argued in his letter of 26.01.94 and during the Oral Proceedings that the specification of the patent and the experimental evidence submitted by the applicant on 09.07.87 during substantive examination (as part of an Appeal against the initial Refusal by the examining division) are sufficient to enable the skilled person to carry out the invention.

In particular the Proprietor has drawn the attention to Experiments 1-3 of said experimental evidence:
Experiment 1: showed uptake by living cells of 4-mer, 13-mer and 14-mer unmodified oligos to the SV40 T-Ag coding region.
Experiment 2: tested in vivo inhibition by 10-, 15-, 20-, 20- and 25-mers (oligos and their methylphosphonate and ethyltriester analogs) to globin coding region. Four were of different length extending into the coding region from the initiating AUG, while the fifth was from later in the sequence. All showed inhibition. The modified oligos were more effective.
Experiment 3: is the earlier Bryan Affidavit work, showing in vitro specific inhibition of translation of the target mRNA of SV40 in the presence of large amounts of

other mRNAs from the cultured monkey cells.

The Proprietor argues that these experiments demonstrate that oligos directed to the coding sequence of a specific target gene can be taken up by living cells, and are capable of inhibiting translation. The results are in accordance with what is taught by the patent, and represents a therapeutic effect where the therapy requires translation inhibition of a specific target gene.

iii. The Proprietor's arguments with respect to the enabling of the invention by the specification of the patent in suit and the further support provided by the the experimental evidence can not be followed.

With respect to the later filed technical information it is noted that this information is in general provided to support the subject-matter claimed in the application. In order to be acceptable under Article 83 EPC the new examples should be carried out with materials directly derivable from the originally filed application or the material should be available (as an obvious choice) to the skilled person by common general knowledge. Furthermore, the OD notes that the information on enabling should be available to the skilled person at the filing or priority date.

iv. The later filed technical information concerned:
Experiment 1: refers to the uptake of unmodified (that is non-stabilized) oligonucleotides by living cells, one being a 14-mer complementary to the coding region of the large T-antigen.
Experiment 2: refers to inhibition of protein synthesis

in living cells: the targeted mRNA is coding for globin, not referred to in the specification the patent in suit. Four of the five oligonucleotides are targeted to the sequence having the initiation codon of translation. The oligonucleotide targeted to an internal sequence is not tested in the phosphotriester form. The addition of DMSO to the medium is not physiological.
Experiment 3: refers to the inhibitition of SV-40 T-antigen protein synthesis in vitro by a non-modified probe using heat treated mRNA.

Thus, only experiment 2 refers to an inhibition of globin synthesis in living cells in tissue culture. The inhibition of globin synthesis and applied antisense oligonucleotides is not referred to in the originally filed application, and is not considered to be an obvious choice.

v. The originally filed application refers to 2 examples:
- Example 1 concerns guidance on how to prepare oligonucleotides in the ethyl phosphotriester form complementary to the coding region of viral T-protein mRNA. The example does not demonstrate actually tested in vitro or in vivo activity for the suggested oligonucleotide probe.
- Example 2 concerns guidance on how to prepare a family of oligonucleotides complementary to the mRNA encoding the beta chain of mammalian follicle stimulating hormone. The specified sequences are expected to be complementary to the sequence starting at nucleotide 97 of the coding sequence, and to inhibit protein synthesis.

vi. The description does neither contain examples about the

therapeutic use of the oligos nor does it describe in detail such a use: adequate instructions in the specification are missing. Although later some data have become available on the succesful use of the antisense oligonucleotides, this does not take away that at the priority date the skilled person was not in the position to carry out the invention without undue difficulty, even when referring to the common general knowledge.

The skilled person is not enabled to achieve the envisaged result without undue difficulty within the whole ambit of the claim containing the "functional" definition of "therapeutic", i.e. the description does not relate to any particular formulation of the therapeutic agent in order to overcome the known problems with penetration of cells of the subject to be treated, stability of the probe, etc.

vii. The Proprietor has argued that the burden to proof that the specification is insufficient lies with the Opponents. In the present case the OD considers that this should not result in an undue burden to the Opponents. The OD considers that, as none of the examples of the specification in conjunction with the drawings, as well as the later filed experiments, have proven that the description has described in detail at least one way of carrying out the invention claimed (Rule 27(1)(e) EPC), the burden of proof lies in the first place with the Proprietor.

In the present case, no enabling method has been proven. The common general knowledge of the skilled person at the priority date includes, as agreed by the different

Parties, a number of oligonucleotides, also falling within the specified length of 14 to 23 bases.
The concept of inhibition of protein synthesis by adding stabilized, complementary oligonucleotides capable of penatrating mammalian cells was known to the skilled person from D2 and D7/D8. In D2 the concept of blocking the ribosome from the template RNA is described, and the concept of specificity (inhibition of some critical proteins) is referred to.
In D7/D8 the inhibition of RNA-translation of a specific cell protein is referred to.

The Proprietor has argued that only an optimizing of the oligonucleotides for the desired use is required, which in itself does not require undue difficulty or inventive step. The Proprietor submitted that the invention lies in the realization of the general principle of the use of antisense oligos according to claim 1 which are directed against the coding region.
This cannot be accepted, however, because of the absence of at least one example demonstrating a therapeutic antisense oligonucleotide. It is also noted that according to Article 52(2)(a) no European patents shall be granted for scientific theories as these theories are not regarded as inventions within the meaning of Art. 52(1) EPC. In the contested patent the hypothesis that the specified antisense oligonucleotide is resulting in therapeutic use is not proven with an example, neither is the way indicated how to optimize in a reliable way an oligonucleotide for this use.

It is agreed that D15 (published in 1992) refers to a succesfull use of ISIS 1082 (a 21-mer phosphorothioate

that inhibits herpes simplex virus types 1 and 2 infections). It is however considered that the skilled person by the contested patent was not put in the position to devise the antisense oligonucleotide ISIS 1082. Furthermore, as mentioned by the Opponents, many more oligonucleotides have been devised (see D47-51) which have proven not to be succesful in therapy.

viii. The requirements following from the legal principle that the patent monopoly should be justified by the technical contribution to the art are not filfilled. The alleged contribution to the art that protein synthesis in vitro may be interrupted by oligos against the coding region does not justify the scope of present claims 1 and 2, as the contribution does not refer to the therapeutic use and thus the invention extends to technical subject-matter not made available to the person skilled in the art by the application as filed.

3.

The grounds of opposition under Article 100(b) EPC in conjunction with Art. 83 EPC do therefore prejudice maintenance of the patent as granted. The specification of the contested patent when referring to a therapeutic agent as claimed in claims 1 and 2 is unsufficiently clear and complete for it to be put into practice by the skilled person without undue burden, taking into account his common general knowledge.

****

Annex 16.10.95 82 903 424.8 UE

OPPOSITION TO EP92574B OF MOLECULAR BIOSYSTEMS INC

PATENTEE'S RESPONSE

LISTING OF CITED REFERENCES

D1 Miller et al	Effects of a Trinucleotide Ethyl Phosphotriester, Gmp(Et)Gmp(Et)U, on Mammalian Cells in Culture (1977) Biochemistry 16: 1988-1996
D2 Miller et al	Biochemical and Biological Effects of Nonionic Nucleic Acid Methyl-phosphonates (1981) Biochemistry 20: 1874-1880
D3 Jayaraman et al	Selective inhibition of Escherichia coli protein synthesis and growth by nonionic oligonucleotides complementary to the 3' end of 16S rRNA (1981) PNAS 78: 1537-1541
D4 Paterson et al cited in B1	Structural gene identification and mapping by DNA mRNA hybrid-arrested cell-free translation (1977) PNAS 74: 4370-4374
D5 Hastie et al cited in B1	Analysis of mRNA populations by cDNA mRNA hybrid-mediated inhibition of cell-free protein synthesis (1978) PNAS 75: 1217-1221
D6 Wallace et al cited in B1	Hybridization of synthetic oligodeoxy-ribonucleotides to Φx174 DNA: the effect of single base pair mismatch (1979) Nucleic Acids Research 6: 3543-3557
D7 Zamecnik et al cited in B1	Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide (1978) PNAS 75: 280-284
D8 Stephenson et al cited in B1	Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxy-ribonucleotide (1978) PNAS 75: 285-288
D9 Tullis	US Patent No: 5,023,243 (1991)

D10 Miller et al — Synthesis of Oligodeoxyribonucleotide Ethyl Phosphotriesters and Their Specific Complex Formation with Transfer Ribonucleic Acid (1974) Biochemistry 13: 4887-4896

D11 Barrett et al — Inhibitory Effect of Complex Formation with Oligodeoxyribonucleotide Ethyl Phosphotriesters on Transfer Ribonucleic Acid Aminoacylation (1974) Biochemistry 13: 4897-4906

D12 E De Clercq — Interferon Induction Increased through Chemical Modification of a Synthetic Polyribonucleotide (1969) Science 1137-1139

D13 Miller et al — Dinucleoside Methyl Phosphonates: Nonionic Analogs of Dinucleotides (1977) Fed. Proc. Abstr. 36: 2231

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D17 Uhlmann et al — Antisense Oligonucleotides: A New Therapeutic Principle (1990) Chemical Reviews 90: 544-562

D18 Tullis et al — Antisense Applications of Synthetic Nucleic Acids: Biotechnology International (1992), 79-88

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D22 Haeuptle et al — Translation arrest by oligodeoxy-nucleotides complementary to mRNA coding sequences yields polypeptides of predetermined length
(1986) Nucleic Acids Research 14: 1427-1448

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D24 Shen cited in B1 — Nucleosides and Nucleotides as Potential Therapeutic Agents
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D25 Blake et al cited in B1 — Inhibition of Rabbit Globin mRNA Translation by Sequence-Specific Oligodeoxyribonucleotides
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(1978) J Mol. Biol. 122: 145-162

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(1985) Biochemistry 24: 6139-6145

D37 Grineva et al — Alkylating Derivatives of Nucleic Acid Components
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(1977) Biochemistry 16: 1239-1250

D41 Maniatis et al — Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, 1982, pages 179-185

Annex 2 16. 10. 95

82 903 424.8

Documents cited in the opposition:

See the combined listing of the patentee: D1-D41

Furthermore, the following art was later cited:

D42 OppoII: Virology: Fraenkel-Conrat et al. Ch.3

D43 OppoII: Cohen & Hogan Sci Am (dec 1994) 50

D44 OppoII: Handbook Degols et al. Ch.9

D45 OppoIV: Nirenberg Methods Enzymol 17

D46 OppoIV: Springgate & Poland Biopolymers 12 (1978) 2241

D47 OppoVI: Stull et al Nucl Ac Res 20 (1992) 3501-8

D48 OppoVI: Barton & Lemoine Br J Cancer 71 (1995) 429

D49 OppoVI: Marcus-S et al Nucl Ac Res 15 (1987) 5749

D50 OppoVI: Monia et al. J Biol Chem 268 (1993) 14514-22

D51 OppoVI: Johansson et al. Nucl Ac Res 22 (1994) 4591-8

D52 Prop : Williamson J Mol Biol 23 (1967)267

Article 106
Decisions subject to appeal

(1) An appeal shall lie from decisions of the Receiving Section, Examining Divisions, Opposition Divisions and the Legal Division. It shall have suspensive effect.

(2) An appeal may be filed against the decision of the Opposition Division even if the European patent has been surrendered or has lapsed for all the designated States.

(3) A decision which does not terminate proceedings as regards one of the parties can only be appealed together with the final decision, unless the decision allows separate appeal.

(4) The apportionment of costs of opposition proceedings cannot be the sole subject of an appeal.

(5) A decision fixing the amount of costs of opposition proceedings cannot be appealed unless the amount is in excess of that laid down in the Rules relating to Fees.

Article 107
Persons entitled to appeal and to be parties to appeal proceedings

Any party to proceedings adversely affected by a decision may appeal. Any other parties to the proceedings shall be parties to the appeal proceedings as of right.

Article 108
Time limit and form of appeal

Notice of appeal must be filed in writing at the European Patent Office within **two months** after the date of notification of the decision appealed from. The notice shall not be deemed to have been filed until after the fee for appeal has been paid. Within **four months** after the date of notification of the decision, a written statement setting out the grounds of appeal must be filed.

Further information concerning the filing of an appeal

(a) The appeal is to be filed with the European Patent Office either at its seat in Munich, at its branch at The Hague or at its Berlin sub-office. The postal addresses are as follows:

(i) European Patent Office
D-80298 Munich
Germany
(Telex: 523656 epmu d)
(Fax: 089/2399-4465)

(ii) European Patent Office
Branch at The Hague
Patentlaan 2
Postbus 5818
NL-2280 HV Rijswijk (ZH)
Netherlands
(Telex: 31651 epo nl)
(Fax: 070/340-3016)

(iii) European Patent Office
Berlin sub-office
D-10958 Berlin
Germany
(Fax: 030/25901-840)

(b) The notice of appeal must contain the name and address of the appellant in accordance with the provisions of Rule 26 (2)(c) EPC, and a **statement** identifying the decision which is impugned and the extent to which amendment or cancellation of the decision is requested (see Rule 64 EPC). The notice of appeal and any subsequent submissions stating the grounds for appeal must be signed.

(c) Notice of appeal must be **filed in writing** (typewritten or printed (Rule 36(2) EPC), by telegram, telex or fax (Rule 36(5) EPC; OJ 6/89 pages 219-225; OJ 9/89 page 396)).

(d) The fee for appeal is laid down in the Rules relating to Fees. The equivalents in the national currencies in which the fee for appeal can be paid are regularly published in the Official Journal of the European Patent Office under the heading "Guidance for the payment of fees, costs and prices".

Europäisches Patentamt
GD 2 16. 10. 95

European Patent Office
DG 2

Office européen des brevets
DG 2

Application/Patent No.: 82903424.8

Minutes of the

[X] oral proceedings
[ ] taking of evidence

before the [ ] Examining Division
[X] Opposition Division

The proceedings were

[X] held in public.
[ ] not public.

Opening and closure of proceedings/taking of evidence: date 21.09.95 time 9.05 date 21.09.95 time 14.20

Present for the European Patent Office:

Chairman: F. FRIEBEL

1st member: P. HOONEN

2nd member: P. GOHLKE

Legally qualified member :

Minute writer : P. GOHLKE

Present for the parties:

1. Applicant/Proprietor:
MOLECULAR BIOSYSTEMS, Inc. represented by
Mr. Armitage, Mr. Walton et al.

identified by authorisation
[ ] dated.....
[X] in the dossier, Sheet No. 57
[ ] general authorisation, No. .....

2. Opponent 1:2:
CHUGAI SEIYAKU K.K. represented by
Mr. Vossius et al.

[ ] dated .....
[X] in the dossier, Sheet No. 10
[ ] general authorisation, No. .....

3. Opponent 2:4:
HOECHST represented by Mr. Thomas
Hiller - Saynovits et al.

[X] dated 12.09.1995
[ ] in the dossier, Sheet No. .....
[ ] general authorisation, No. .....

4. Opponent 5:
GEN-PROBE, Inc. represented by
Mr. Hoon et al.

[ ] dated.....
[X] in the dossier, Sheet No. 44
[ ] general authorisation, No. .....

5. Other opponents, see Annex 1.

[X] The Chairman confirmed that the person(s) not appearing had been duly summoned.

The essentials of the oral proceedings/taking of evidence and the relevant statements of the parties are set out in the annexes (Form 2906).

16. 10. 95

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Annex 1

6. Opponent 6:

CIBA-GEIGY ... represented by ... Mr. Maschio (European patent Attorney) et al.

☐ dated .....
☐ in the dossier, Sheet No. .....
☐ general authorisation, No. .....

7. Opponent 7:

GENTA Inc. ... represented by Mrs. Kolb ... (European patent Attorney) et al. .....

☐ dated .....
☐ in the dossier, Sheet No. .....
☐ general authorisation, No. .....

- Opponent I (Hybridon, Inc.) has already withdrawn his opposition with letter of 25.11.94 and opponent III (Applied Biosystems Inc.) has indicated not to be present at the Oral Proceedings (see file page 177), thus only opponents II (Chugai Seiyaku K.K.), IV (Hoechst), V (Gen-Probe, Inc.), VI (Ciba-Geigy) and VII (Genta Inc.) were present.

- The Chairman declared the Oral Proceedings open.

- The submissions as filed in the written procedure are maintained. All late filed documents are acceptable (a list of the later filed documents D42-D52 made by the Opposition Division was given to the parties: see Annex 2). In its letter dated 18.08.95, Opponent VI requested that the patent be revoked in full on the grounds of lack of novelty over citation D33.

- Sufficiency of the disclosure was first dealt with.
The Chairman gave word first to Mr. Moonen (first examiner of the Opposition Division) who briefly summarized the objections raised under article 83 EPC by opponents II, V, VI and VII.

Mr. Armitage (Patentee's representative) was asked to reply thereto; he gave word to his collaborator Mr. Walton.
Mr. Walton firstly stated that claim 1 provides a methodology of developing therapeutic agents which comprises the determination of a target sequence (= appropriate sequence of the mRNA specific for the protein to be inhibited) and then the manufacture of an oligonucleotide complementary to a portion of the coding region of the chosen mRNA. Thus after having chosen a targeted sequence, the skilled man has the possibility to develop an oligonucleotide lead compound by routine empirical research, thus without the exercise of inventive effort, by varying the length from 14 to 23

bases, trying an RNA or DNA sequence, varying the way of stabilization, etc., as suggested by claim 1, and then optimize. According to Mr Walton the invention does obviously work, this is supported for example by D15 disclosing that "several compounds are currently in preclinical development" (cf. p.346); "topical application of ISIS 1082 ... cured the infection" (cf. p.366) and that impressive progress has been made (see conclusions).

The principle of the invention is "try any oligonucleotide fullfilling the conditions of claim 1 and see if it has a therapeutical effect". The Patentee is not interested in ineffective antisense oligonucleotides. The invention should not be restricted to the examples as there is a technical concept which fits for generalisation (this was not the case in T435/91 or T409/91). In their letters the opponents have raised a doubt upon the effectiveness of the examples described in the patent. Mr. Walton stated that burden of proof should lie on the opponent. As none of them have filed any experimental data, it is supposed that the examples do work.

Furthermore, oligonucleotides referred to by the opponents in documents D47-D51 and which are incapable of blocking translation are irrelevant for the Patentee. Citations D47 to D51 were discussed in detail and Mr. Walton mentioned that these citations also provide other oligonucleotides which are effective.

After a short break word was given to the opponents. In summary, Mr. Vossius (Opponent II) alleged that the guidance to the skilled person, provided by the specification of the patent is not sufficient to carry out the invention, at least not without undue experimentation and not within the whole area that is claimed. Claim 1 is merely a strategy claim without a fully self-sufficient technical concept.

Mr. Müller-Saynovits (Opponent IV) agreed with Mr. Vossius.

Mr. Kacian, a technical expert of Opponent V calculated the multitude of possibilities to found out one working oligonucleotide. He obtained, in very generous conditions, the number of 26.475.000 experimentations to be carried out. Thus a routine empirical experimentation as suggested by the invention is impossible.

Mr. Maschio (Opponent VI) also agreed with Mr. Vossius and furthermore he summarized the points raised in his letter dated 18.08.95.

Mrs. Kolb (Opponent VI) agreed with Mr. Vossius in that the patent is a strategy not fully self-contained. Dr. Arnold, a technical expert, discussed in detail the comparative tests later filed by the Patentee; as already stated by the opponents in their letters, these tests fail to provide any results illustrating the therapeutic effectiveness of said stabilized oligonucleotides. Dr. Arnolf pointed out the difficulty for the oligonucleotide not to be stripped-off by the ribosome and the variability of the results in erythroleukemia assay.

After lunch-break, Mr. Walton did not provide any new argument to the one he gave at the beginning of the Proceedings or in its letter dated 26.01.94.

- The Opposition Division reached the decision to revoke the patent under Article 102(1) EPC. The subject-matter of claims 1 and 2 as granted does not satisfy the requirements of the European Patent Convention because of insufficiency of disclosure. There is a failure to obtain a therapeutic agent: it is considered that the description is unsufficiently clear and is also incomplete for it to be put into practice by the skilled person without undue burden, taking into account his common general knowledge at the date of priority.

The Oral Proceedings were closed.

16. 10. 95

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Annex 2

D42 OppoII: Virology: Fraenkel-Conrat et al. Ch.3

D43 OppoII: Cohen & Hogan Sci Am (dec 1994) 50

D44 OppoII: Handbook Degols et al. Ch.9

D45 OppoIV: Nirenberg Methods Enzymol 17

D46 OppoIV: Springgate & Poland Biopolymers 12 (1978) 2241

D47 OppoVI: Stull et al Nucl Ac Res 20 (1992) 3501-8

D48 OppoVI: Barton & Lemoine Br J Cancer 71 (1995) 429

D49 OppoVI: Marcus-S et al Nucl Ac Res 15 (1987) 5749

D50 OppoVI: Monia et al. J Biol Chem 268 (1993) 14514-22

D51 OppoVI: Johansson et al. Nucl Ac Res 22 (1994) 4591-8

D52 Prop : Williamson J Mol Biol 23 (1967)267

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Nachnahme DM Pf

Wert DM

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Hoffmann, Eitle & Partner,
Patentanwälte,
Postfach 81 04 20
D-81904 München
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Vossius, Volker, Dr.
Dr. Volker Vossius,
Patentanwaltskanzlei-Rechtsanwaltskanzlei
Holbeinstrasse 5
D-81679 München
ALLEMAGNE

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Tagesstempel: Frankfurt am Main, z, 18.10.95-8, 08

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17. Okt. 1995
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(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 092 574 B1**

(12) EUROPEAN PATENT SPECIFICATION

(45) Date of publication of patent specification: **29.04.92**

(51) Int. Cl.5: **C07H 21/02**, C07H 21/04, C12N 15/00, C12Q 1/68, C12P 21/00, C12P 19/34

(21) Application number: **82903424.8**

(22) Date of filing: **08.10.82**

(86) International application number:
PCT/US82/01447

(87) International publication number:
WO 83/01451 (28.04.83 83/10)

The file contains technical information submitted after the application was filed and not included in this specification

(54) OLIGONUCLEOTIDE THERAPEUTIC AGENT AND METHODS OF MAKING SAME.

(30) Priority: **23.10.81 US 314124**

(43) Date of publication of application:
02.11.83 Bulletin 83/44

(45) Publication of the grant of the patent:
29.04.92 Bulletin 92/18

(84) Designated Contracting States:
AT BE CH DE FR GB LI NL SE

(56) References cited:
GB-A- 2 068 971
US-A- 4 237 224
US-A- 4 321 365

PNAS USA, Vol. 75 (1) (1978), 285-288

Affidavit of Dr. Robert N. Bryan (06-02-1984)

Angew. Chem. Int. Ed. Vol. 9 (1970) No. 9, p. 678-688

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EP 0 092 574 B1

Biochemistry Vol. 24(22) (1985), 6139-6145

PNAS USA Vo.. 83 (1986), 4143-4146

N. WALLACE ET AL. NUCLEIC ACIDS RESEARCH, VOLUME 6, NUMBER 11, 1979, PAGES 3543-57

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N. HASTIE ET AL, PROC. NATL: ACAD. SCI., USA, VOLUME 75, NUMBER 3, MARCH 1978, PAGES 1217-21

N. ALVARADO-URBINA ET AL, SCIENCE, VOLUME 214, 16 OCTOBER 1981, PAGES 270-74

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PROC. NATL: ACAD, SCI, USA, vol. 75, no. 1, January 1978 P.C. ZAMECNIK et al.: "Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide", pages 280-284

Description

This invention relates to a therapeutic agent for selectively blocking the translation of mRNA into a targeted protein by utilizing hybridization techniques of the type having messenger ribonucleic acid attached to oligonucleotides for in vivo protein synthesis regulation.

In the field of pharmacology, the use of therapeutic agents has long been recognized as an effective way to control diseases. Such agents are often used in treating bacterial or viral infection, chemical imbalances and the like, to cure, or at least mitigate, the diseased state. Although researchers occasionally discover new therapeutic agents after major breakthroughs have elucidated the molecular basis of a disease, more often they must rely on observing for antibiosis or modifying the chemical structures of functionally related chemicals.

With respect to antibiotic agents, some are quite effective at the outset, but over time many organisms become resistant or totally immune to their action. Additionally, very few effective antiviral agents have ever been developed, and without explicit, detailed knowledge of an infecting organism's physiology, the development of new operative agents remains haphazard.

In PNAS USA vol. 75, No. 1 (1978), pages 280 to 284 a particular tridecamer complementary to 13 nucleotides of the 3' and 5' reiterated terminal sequence of Rous Sarcoma virus 35 RNA is described. The tridecamer is observed to inhibit the virus production in chick embryo fibroblast tissue cultures. It is contemplated that the tridecamer might hybridize with the repetitive terminal sequence of the RNA and might interfere with the circularization step of the pro-virus DNA.

A corresponding tridecamer is described in PNAS USA vol. 75, No. 1 (1978), pages 285 to 288. The document refers to the ability of the tridecamer to prime virus RNA and to inhibit protein synthesis from the virion RNA. It is speculated that the inhibition might be due to hybridization of the tridecamer near potential initiation sites for viral protein synthesis.

In PNAS USA vol. 75, No. 3 (1978), pages 1271 to 1221 and PNAS USA vol. 74, No. 10, (1977), pages 4370 to 4374 in vitro methods of structural gene identification and mapping by DNA-mRNA hybrid-arrested cell-free translation are described. In the method DNA fragments of 500 to 1000 bases in length were hybridized with mRNA coding for known proteins at a temperature of 65 °C for 20 min. or 48 °C for 2 hr. It was found that translation of the hybridized regions of mRNA was reversably blocked at these conditions.

Angewandte Chemie International Edition, vol. 9 (1970), No. 9, pages 678 to 688 describes the use of modified nucleosides or nucleotides as antimetabolic agents for inhibiting the nucleic acid metabolism and particularly the synthesis of virus induced cDNA. The modified nucleosides or nucleotides are competing with their unmodified analogues for specific binding sites and positions within complex structures thereby blocking the physiological functions of the resulting products. It is further considered that the compounds may have immunological effect since DNA synthesis inhibitors are effective in suppressing the proliferation of sensitized lymphocytes and cell-mediated immunity. In this context reference is also made to oligonucleotides on the basis of the observation that antibody stimulation occurred with a gross mixture of DNA digest, the optimal size of the oligomers being indicated as tri- to hexanucleotides. It is speculated that the modified oligonucleotides would compete with their naturally occurring analogues for the protein binding sites, thereby blocking antibody production particularly in auto-immune diseases.

Finally, in Biochemistry, vol. 16, No. 9 (1977), pages 1988 to 1996 the preparation of a particular modified trinucleotide is described, which is complementary to a three base sequence common to the aminoacid accepting stem of most tRNAs. The document reports in vitro experiments, showing that the triplet is bound to tRNAs and inhibits amino acylation at a rate of 39% at 37 °C. It was further shown that the triplet passes into the cells in vivo unspecificially reducing peptide synthesis for an interval of about 24 hours.

No therapeutic agent has up to now been described in the prior art, by means of which it would be possible to selectively block the translation of mRNA into a particular, targeted protein without effecting the synthesis of other proteins.

Thus, there exists a definite need for a therapeutic agent that is versatile and inexpensive and yet both extremely specific and effective. The present invention fulfils these needs.

SUMMARY OF THE INVENTION

The present invention provides a methodology of identifying and constructing therapeutic agents for use in living organisms that substantially reduces the uncertainty surrounding the development of new antagonists, significantly increasing the scope of materia medica. Moreover, the agent construction of the present invention lends itself readily to simple manufacture, even in large quantities, is extremely effective in use, and attains its improved results without undue cross-reactions.

According to the invention a stabilized oligonucleotide, preferably in a phosphotriester

form, is provided having a base sequence substantially complementary to a portion of messenger ribonucleic acid coding for a biological component of an organism. Due to the complementary nature of the oligonucleotide and the messenger ribonucleic acid, the two components can readily hybridize under appropriate conditions to control synthesis of the organism's biological component and, if the protein is vital to the organism's viability, to act as an antibiotic.

A method, in accordance with the present invention, of developing therapeutic agents may typically include the steps of: providing a base sequence of an organism's nucleic acid that contains at least a portion of the genetic information for a biological component of the organism, and synthesizing an oligonucleotide the sequence of which is derived from the base sequence, for subsequent hybridization with the messenger ribonucleic acid specific for the biological component. The biological component may be a vital protein, or simply a hormone such as the gonadotropin, follicle stimulating hormone. The order of the base sequence may be determined from deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), preferably messenger ribonucleic acid (mRNA). Alternatively, the desired oligonucleotide base sequence may be determined from the biological component's sequence, as when the biological component is a protein. The oligonucleotide of the invention has fourteen to twenty-three bases, and for increased stability, is transformed to a more stable form, such as a phosphotriester form, to inhibit degradation during use.

To produce large quantities of the oligonucleotide, it may be synthesized chemically, such as in automated machines, or inserted into a plasmid, such as pBR322, for cloning. The plasmid insertion may be accomplished with linker base sequences, such as GATTCGAATC or CTAAGCTTAG, which are susceptible to degradation by Hind III restrictive nuclease or Alu I restriction nuclease. When the order of the base sequence has not been determined, the base sequence can be cloned and then cross-hybridized against messenger ribonucleic acid from the other sources to remove base sequences non-specific to the target.

Another aspect of the present invention is a method of selectively controlling activity of one or more biological components in a cell without substantially interfering with the activity of other biological components in the cell. The method includes the steps of forming an oligonucleotide having a base sequence substantially complementary to a portion of mRNA coding for the specific biological component, and introducing the oligonucleotide into the cell for hybridization with the selected mRNA. This causes blocking of the translation of the mRNA into protein. The oligonucleotide has fourteen to twenty-three bases. The target mRNA can code for a protein, such as the hormone, follicle stimulating hormone. This hormone has an alpha and beta chain, and the oligonucleotide should be specific for the mRNA coding for the beta chain to avoid cross-reacting with other gonadatropin mRNA. A suitable oligonucleotide base sequence would be AC-CACGCGR$_1$CCR$_2$ATGACGATGTG, wherein R_1 is G or T and R_2 is also G or T.

In accordance with another aspect of the present invention, a method is provided for inhibiting the infection of a host organism by a foreign organism. This method entails isolating a base sequence containing at least a portion of the genetic information coding for a vital protein from the foreign organism's nucleic acid; synthesizing an oligonucleotide, the order of which is derived from the base sequence and substantially complementary to the messenger ribonucleic acid coding for the protein; and treating the foreign organism with an effective amount of the oligonucleotide to hybridize with a portion of the messenger ribonucleic acid and block translation of the protein. The oligonucleotide, which can be a deoxyribonucleotide, is transformed to a more stable form, such as a phosphotriester form, to inhibit degradation, and the order of the sequence determined prior to its synthesis. Further, to increase the oligonucleotide's specificity, it may be cross-hybridized against mRNA from different organisms, such as the host organism, to remove non-unique oligonucleotide sequences.

It will be appreciated from the foregoing that the present invention satisfies a long existing need for improved methods of developing therapeutic agents for use in living organisms, and represents a significant advance over previously available methods, principally because it is very versatile, and yet provides a very specific agent against a biological component. Other aspects and advantages of the invention will become apparent from the following more detailed description taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a flow diagram showing the central dogma of molecular biology;

FIG. 2 is a flow diagram showing normal translation of messenger ribonucleic acid (mRNA) into T protein, as well as a synthetic oligonucleotide of the present invention blocking translation of the T protein;

FIG.3 is a list of the viral deoxyribonucleic acid (DNA) code specific for SV-40 T protein, and the related mRNA and oligonucleotide;

FIG.4 is a flow diagram showing the construction of high yield plasmids containing the T protein oligonucleotide;

FIG.5 is a flow diagram showing the use of restriction nucleases to cleave the plasmids to give a purified T protein oligonucleotide;

FIG. 6 is a flow diagram showing the treatment of a DNA sequence to form DNA polyphosphotriesters; and

FIG. 7 is a chart showing the partial amino acid sequence of follicle stimulating hormone, as well as the predicted mRNA sequence and related oligonucleotide family.

DETAILED DESCRIPTION OF THE INVENTION

Referring now to the drawings, and particularly to FIGS. 1 and 2, there is shown the so-called "central dogma" of the molecular biology of life. Basically, it is now accepted that deoxyribonucleic acid (DNA) carries the genetic code for almost all living organisms. The code exists in the form of an organized sequence of four nucleotide bases attached to a phosphorylated, deoxyribose sugar backbone. Generally, DNA exists in the form of a double strand helix formed of two oppositely directed strands, which are held together in an opposing manner by various weak forces.

A primary constituent of these weak forces are the so-called hydrogen bonds that exist between nuclectides on the opposing strands. The four bases, adenine (A), cytosine (C), guanine (G), and thymine (T), form hydrogen bonds generally in only one fashion: A with T and C with G. Thus, by knowing the sequence of one strand, the sequence of the second strand can be readily determined.

Another aspect of the central dogma is that proteins are produced indirectly from the DNA strand, through messenger ribonucleic acid (mRNA). Apparently, mRNA, which has the same structure as single stranded DNA except with a ribose backbone and with uracil (U) replacing thymine, is transcribed directly from one DNA strand and has an essentially opposite base sequence, i.e., if a DNA strand sequence is 5 ...ACGT...3' the transcribed mRNA sequence is 3'...UGCA...5'.

An additional aspect of the central dogma relates to the translation of mRNA into proteins. Briefly, excluding initiation sites and the like, each three nucleotide base grouping (triplet code) codes for one amino acid of a protein. Therefore, by knowing the mRNA sequence of a protein, its amino acid sequence can generally be determined. However, the reverse is not true, that is, knowing the amino acid sequence does not guarantee precise knowledge of the mRNA sequence. This stems from the fact that there exist 64 (4^3) possible triplet codes, yet there exists only about twenty amino acids, allowing some amino acids to have multiple triplet codes.

The similarity in the structure of DNA and mRNA strands creates interesting effects. Most notably, if complementary DNA and RNA strands exist contemporaneously in a solution, under certain established conditions the strands can anneal, forming hybrids. One important factor in proper annealing is the melting temperature, which can be calculated according to Britten et al, Methods of Enzymology 29:363 (1974).

In accordance with the present invention, a synthetic oligonucleotide having a base sequence of 14 to 23 bases capable of substantially matching that of a chosen mRNA is provided for hydridization with that mRNA. Once such a hybrid exists, the translation of the mRNA into protein becomes significantly inhibited. If the inhibited protein is vital to an organism's survival, the organism's viability, i.e., either growth or continued life, is jeopardized. Importantly, the oligonucleotide can be designed specifically for the mRNA coding for just one protein, and should not cross-react with mRNA for other proteins.

The method of developing the oligonucleotide entails basically two steps. As described more fully below, a possible first step is to determine the appropriate sequence of the mRNA specific for the protein to be inhibited, and a second step is to manufacture an oligonucleotide complementary to the mRNA. Once made, the oligonucleotide is treated into e.g. a phosphotriester form for increased stability.

A variety of techniques exists for determining nucleic acid base sequences. In many instances the sequence of the mRNA or the gene have been determined and published in the biochemical literature. In fact, researchers have determined the complete nucleotide sequence for the SV-40 virus (Reddy et al, Science 200:494 (1978)). As is well known, an alternative method entails isolating and purifying mRNA in sufficient quantities to permit sequencing studies, but this can prove difficult due to the relative instability and, in some cases, extreme rarity of many mRNA sequences.

Still another method for determining nucleic acid base sequences requires resolving the amino acid sequence from the target protein. After determining the amino acid sequence of the target protein in purified form, a sequential degradation utilizing commercially available protein sequences (e.g. from Beckman Instruments, Fullerton, California) can be used to provide the amino acid sequence. Once this has been obtained, knowledge of the triplet code can be applied to give prospective base sequences. An example of such a process for the hormone glucagon can be found in Tullis et al,

Biochemical and Biophysical Research Communications 93:941 (1980).

Once the sequence of the appropriate nucleic acid and the desired mRNA sequence have been determined, an oligonucleotide, such as deoxyribonucleotide, complementary to the mRNA can be constructed. A number of synthetic techniques are known, most typical is the diester approach described by Agarwal et al, Nature 227: 27 (1970), and oligonucleotide synthesizers may be purchased commercially from Vega Biochemicals, P.O. Box 11648, Tucson, Arizona and Biologicals, Inc., Toronto, Canada.

If the desired oligonucleotide sequence is unknown, a suitable oligonucleotide can be prepared as follows. After isolating mRNA from a target organism, multiple copies are made, preferably in the form of DNA, so-called copy DNA (cDNA). This cDNA is then cross-hybridized against mRNA isolated from other organisms, and any cDNA hybridizing is removed. The remaining cDNA is specific only to the target organism, and can serve as the therapeutic agent.

In order to obtain a high degree of specificity, an oligomer of 14 to 23 bases is constructed. Although shorter sequences would work, these sequences provide higher specificity. This can readily be seen mathematically. Whereas a ten unit polymer chosen from four bases can have 4^{10} - (1,048,576) random combinations, a 20-unit polymer has 4^{20} random combinations, which equals 1.09×10^{12} (1,090,000,000, 000).

In spite of the added difficulty in making oligonucleotides of twenty units in comparison to ten bases, it is warranted because the exponential increase in complexity reduces undesirable cross-reactivity. It has been estimated that a mammalian cell contains about 2×10^8 nucleotides of RNA complexity or, in other words, approximately 200 million nucleotides of unique sequence mRNA, which is equivalent to about 30,000 mRNA sequences. The probability that one of those sequences contains a randomly chosen 20-unit polymer is approximately one in fifty-five hundred. In comparison, a ten-unit polymer has about a one hundred and ninety to one chance for random cross-reaction.

The present invention is illustrated by, but not limited to, the following examples.

EXAMPLE I

SV-40 virus manufactures a vital protein commonly known as the "T protein" or "T antigen protein". As noted earlier, the complete genetic code for the SV-40 virus has been determined, and it is known that residues 5091 to 5071 on the viral genome code for a portion of the T protein mRNA. The sequence of these residues, the viral T protein mRNA sequence, and the designed T protein specific oligonucleotide are shown in Fig. 3. In this case, the T protein specific oligonucleotide is complementary to the viral T protein mRNA, and identical to the portion of the viral DNA code.

Prior to testing the effectiveness of the T protein specific oligonucleotide in vivo, the oligonucleotide can be mixed with total mRNA from an organism to check for cross-reactivity. If it hybridizes, then a different portion of the viral genome coding for the T protein should be utilized. Otherwise, the oligonucleotide is ready for further testing.

Further testing requires growth of S-40 virus. For purposes of these experiments, SV-40 virus is grown and titered in African green monkey cells, such as the cell line BSC-1, according to Hopps et al, Journal of Immunology 91:416 (1963). The identity of the virus can be confirmed by the following methods:

a) checking for tumor production after innoculation of the newborn hamsters with the virus;

b) neutralization of the virus by anti-SV-40 antiserum; and

c) reaction of the infected cells with anti-SV-40 T antigen directed antibodies prepared by standard techniques.

The isolation of SV-40 mRNA can be accomplished as follows. Total RNA is first obtained by the guanidine hydrochloric acid extraction procedure using glassware previously baked and treated with diethylpyrocarbonate to remove traces of RNase as taught by Cox et al, Methods in Enzymology 12B:120 (1968). The A+ RNA is isolated on oligo-dTcellulose, which can be obtained from Collaborative Research, Waltham, Massachusetts, or P. L. Biochemicals, Inc., Milwaukee, Wisconsin, utilizing the technique described by Bantle et al, Analytical Biochemistry 72:413 (1976). The RNA fractions are assayed for purity and intactness by electropheresis according to the method described in Bailey et al, Analytical Biochemistry 70:75 (1976). Also, the RNA can be assayed for translatability in the wheat embryo in vitro system described in Marcus, et al, Methods in Enzymology: 30:749 (1974). The in vitro translation products are monitored on sodium lauryl sulphate 9% polyacrylamide gels as described in Laemmeli, Nature 227:680 (1970).

This purified A+ mRNA containing the viral mRNA sequences can hybridize to the synthetic oligonucleotide at 37°C in 0.5 M sodium phosphate buffer, pH 6.8, containing 0.2% sodium lauryl sulphate. Solutions containing about 1 mg A+ mRNA and synthetic oligonucleotide at a concentration of about 100 ug/ml are heated to 100°C for 1-2 minutes, then cooled to 37°C and allowed to anneal. The extent of the hybridization reaction as a func-

tion of time may be monitored on a gel filtration column.

Actually, while any theoretically suitable temperature may be used for the hybrid formation, temperatures ranging from 0°C to about 80°C provide for good hybridization, but preferred temperatures range from about 10°C to about 40°C. Generally, the optimal annealing temperature for the formation of specific hybrids is believed to be about 20°C to 25°C below their melting temperature. Synthetic oligonucleotides operating at 37.5°C should thus be designed on the basis of their base sequence and length, such that the melting temperature is between about 57°C and 62°C when tested under approximately physiological conditions.

For hybridization testing the ratio of the synthetic oligonucleotide to its mRNA complement is generally about 30:1. Lower ratios are acceptable, however, sequences below about 3:1 can cause lower hybrid formation. Control reactions utilizing yeast RNA or globin mRNA can be used, and should show no detectable hybrids, indicating hybridization specificity only to SV-40 mRNA. Also, thermal denaturation profile studies and comparison of the kinetics of hybridization can confirm that the synthetic oligonucleotide reacts only with SV-40 mRNA sequences.

Once it is shown that the oligonucleotide hybridizes to the isolated SV-40 mRNA, in vitro translation tests can be attempted utilizing the wheat embryo system (described previously) to show that the hybrid is not translated. Basically, upon introduction of SV-40 mRNA into the wheat embryo system, the system produces large T antigen protein. However, when an equal amount or more of synthetic oligonucleotide is also added to the system, T antigen protein synthesis can be substantially inhibited, without interference with synthesis of other SV-40 proteins whose mRNA was also introduced.

Testing of the oligonucleotide in vivo can be accomplished-by adding the oligonucleotide to cultures of cells infected with SV-40. Synthesis of T antigen protein should be inhibited significantly in about six hours, and SV-40 growth should be strongly inhibited within about 24 hours. The growth of control cultures should be largely unaffected.

The synthetic oligonucleotide of the present invention may be mass produced according to common cloning techniques, such as those developed in the art to clone the gene for proinsulin. Alternatively, the oligonucleotide can be chemically synthesized in commercially available equipment (described previously). Briefly, the cloning method entails enzymatic insertion of the oligonucleotide into a bacterial gene carried on a larger piece of DNA, known as a plasmid. The plasmid can be incorporated into a suitable host bacteria, and multiple copies made as the bacteria multiply as in Boyer and Cohen, U. S. Patent No. 4,237,224.

More particularly, and with reference to FIGS. 4 and 5, the cloning plasmid designated as pBR322, available from Bethesda Research Labs, Inc., Rockville, Maryland, can be used to mass produce the T protein specific oligonucleotide. Using standard techniques, the oligonucleotide is converted to double stranded form and then a terminal $5'PO_4$ is added to each of the 5' termini with polynucleotide kinase to permit subsequent joining through T-4 ligase. The reaction conditions for forming the 5' termini can be found in Richardson, Progress in Nucleic Acids Research 2:815 (1972).

After purification of the double stranded oligonucleotide by chromatography on hydroxylapatite columns, it is inserted into the plasmid. Because the oligonucleotide is blunt ended, the plasmid should not have uneven or "sticky" ends. To remove sticky ends from the plasmid, S1 nuclease or other single strand specific nucleases can be utilized. A general description of methods for using restriction nucleases can be found in Nathans and Smith, Annual Review of Biochemistry 44: 273 (1975).

For best results, a linker system between the oligonucleotide and the plasmid can be utilized, specifically a linker having both Hind III and Alu I enzymatic cleaving sites. As seen in FIG. 4, one such linker has a sequence: 5'... CTAAGCTTAG...3'. This sequence represents a double stranded, bisymmetric molecule containing a recognition sequence both for Alu I (AGCT) and for Hind III (AAGCTT). Utilizing DNA ligase under standard conditions, this molecule can be ligated to the oligonucleotide to form linker-oligonucleotide-linker molecules. Similarly, the linked oligonucleotide can be introduced into linearized blunt-ended, Hind III cleaved pBR322 carrier molecules.

After ligation, the plasmid has resumed its covalently closed circular configuration with the linker-oligonucleotide incorporated, all of which is shown in FIG. 5 as pT-protein oligonucleotide. The recircularized plasmid is then used to transform a suitable bacterial host such as E. coli. The methods for transformation and selection of transformants are known in the art and described in detail in Cohen and Boyer, U.S. Patent No. 4,237,224.

Once the transformed bacteria containing the ligated plasmid p-oligonucleotide have been grown to high density and produced large amounts of the ligated plasmid, the oligonucleotide is ready for purification. After the plasmid has been removed from the mature cells, the plasmid is treated with appropriate restriction endonucleases. As illustrated in FIG. 5, the plasmid is first cleaved with Hind III

to give various by-products, including linker-T-protein-oligonucleotide-linker and fragments of the original plasmid. These are readily separated utilizing gel electrophoresis or high pressure liquid chromatography. Further cleavage of the isolated linker-oligonucleotide-linker with the endonuclease Alu I yields pure double stranded oligonucleotide and partially degraded linker. These can also be separated based on their size differences.

As shown in FIG. 6, the oligonucleotide can then be modified to a nuclease resistant phosphotriester form utilizing the reaction described in Miller et al, Biochemistry 16:1988 (1977). Basically, the oligonucleotide is first acylated using 50% acetic anhydride-pyridine during an overnight incubation period. The product is precipitated and isolated from ether. The phosphotriester can then be formed utilizing 30% ethanol in anydrous 2, 6 lutidine (30%), NN-dimethyl formamide (30%) and p-toluene sulfonyl chloride (17%), and reacting for about 6 hours. The protecting acetyl groups are then hydrolyzed by the addition of 0.5 volumes of concentrated ammonium hydroxide, followed by incubation for about 1 hour at 55°C, The final oligonucleotide product in the ethyl phosphotriester form can then be isolated on paper chromatography or high pressure liquid chromatography.

It is believed that transforming the oligonucleotide to a phosphotriester form will improve the oligonucleotide's stability in vivo due to an enhanced resistance against various degradative enzymes. However, the oligonucleotide will eventually degrade because of spontaneous de-ethylation, which leaves the molecule unprotected. Indeed, by controlling the initial level of ethylation, the in vivo degradation rate can be controlled. A further advantage of a phosphotriester form is believed to be an increase in the oligonucleotide's ability to penetrate a cell membrane.

EXAMPLE 2

A synthetic oligonucleotide capable of inhibiting the synthesis of follicle stimulating hormone (FSH), a protein hormone produced by the pituitary that functions in the maturation of ova in females and sperm cells in males, can also be constructed. It is known that FSH is composed of two chains, alpha and beta, the amino acid sequence of which has been determined for several animal species. Interestingly, the alpha chain of FSH is common to other gonadotropic hormones, including thyroid stimulating hormone, luteinizing hormone, and chorionic gonadotropin, while the beta chain varies. Therefore, to selectively shut off the synthesis of FSH without substantially affecting the other gonadotropins, the oligonucleotide must be specific for the mRNA coding for the beta chain.

The sequence of the beta chain amino acids 32 through 40 is shown in FIG. 7. As discussed earlier, it is possible to predict the mRNA base sequence for these amino acids, although not with absolute certainty. The points of uncertainty are indicated by the letter "X" in the predicted mRNA sequence. Thus, the resultant oligonucleotide family consists of eight possible 26 base sequences; the potential alternate bases are shown in parentheses below the primary base sequence.

By beginning with the projected mRNA sequence for the 33rd through 40th amino acids, it can be seen that four different 23 base oligonucleotides exist that could correspond to the FSH mRNA. The sequences could be as follows, reading from the 5' end: GTGTAGCAGTAGCCGGCGCACCA, GTGTAGCAGTATCCGGCGCACCA, GTGTAGCAGTAGCCTGCGCACCA, and GTGTAGCAGTATCCTGCGCACCA.

One of these four sequences should be precisely correct and thus able to hybridize fully with the FSH mRNA. To determine the best sequence, a hybridization test against FSH mRNA, with subsequent purification on hydroxylapatite or other suitable column, can be performed as previously described. Once the best sequence has been determined, it is placed in a plasmid or chemically synthesized, as described above, for bulk synthesis. This oligonucleotide should substantially inhibit the synthesis of FSH in vivo.

From the foregoing, it will be appreciated that the present invention provides a systematic method of designing new therapeutic agents for use in living organisms and that this method is versatile and inexpensive. Further, the oligonucleotide produced in accordance with the present invention is extremely effective and specific, enabling selective control of protein synthesis in a living organism.

While several particular forms of the invention have been illustrated and described, it will be apparent that various modifications can be made without departing from the spirit and scope of the invention. Accordingly, it is not intended that the invention be limited, except as by the appended claims.

Claims

Claims for the following Contracting States : BE, CH, DE, FR, GB, LI, NL, SE

1. Therapeutic agent for selectively blocking the translation of mRNA into a targeted protein, comprising a stabilized oligonucleotide of 14 to 23 bases having a base sequence substantially complementary to a portion of the coding region of the mRNA coding for said targeted protein.

2. Therapeutic agent according to claim 1, characterized in that the oligonucleotide is in a phosphotriester form.

Claims for the following Contracting State : AT

1. A method for preparing a therapeutic agent for selectively blocking a translation of mRNA into a targeted protein comprising the manufacturing of a stabilized oligonucleotide of 14 to 23 bases having a base sequence substantial complementary to a portion of the coding region of the mRNA coding for said targeted protein and formulating the same into a therapeutic agent.

2. A method for preparing a therapeutic agent according to claim 1, characterized in that the oligonucleotide is prepared in a phosphotriester form.

Revendications

Revendications pour les Etats contractants suivants : BE, CH, DE, FR, GB, LI, NL, SE

1. Agent thérapeutique qui bloque d'une manière sélective la traduction de l'ARNm d'une protéine cible, comportant un oligonucléotide stabilisé de 14 à 23 bases dont une séquence de bases est en grande partie complémentaire d'une fraction de la région codante de l'ARNm codant pour ladite protéine-cible.

2. Agent thérapeutique selon la revendication 1, caractérisé en ce que l'oligonucléotide est sous forme de phosphotriester.

Revendications pour l'Etat contractant suivant : AT

1. Procédé de fabrication d'un agent thérapeutique qui bloque d'une manière sélective une translation d'ARNm d'une protéine cible, comprenant la préparation d'un oligonucléotide stabilisé de 14 à 23 bases dont une séquence de bases est en grande partie complémentaire d'une fraction de la région codante de l'ARNm codant pour ladite protéin cible et la formulation du même en un agent thérapeutique.

2. Procédé de fabrication d'un agent thérapeutique selon la revendication 1, caractérisé en ce que l'oligonucléotide est préparé sous forme de phosphotriester.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : BE, CH, DE, FR, GB, LI, NL, SE

1. Therapeutisches Agens zum selektiven Blokkieren der Translation von mRNA in ein Zielprotein, welches ein stabilisiertes Oligonukleotid mit 14 bis 23 Basen umfaßt, das eine Basensequenz aufweist, die im wesentlichen komplementär zu einem Bereich der kodierenden Region der mRNA ist, welche für das Zielprotein kodiert.

2. Therapeutisches Agens nach Anspruch 1, **dadurch gekennzeichnet**, daß das Oligonukleotid in Form eines Phosphotriesters vorliegt.

Patentansprüche für folgenden Vertragsstaaten : AT

1. Verfahren zum Herstellen eines therapeutischen Agens zum selektiven Blockieren einer Translation von mRNA in ein Zielprotein, welches das Herstellen eines stabilisierten Oligonukleotids mit 14 bis 23 Basen mit einer Basensequenz, die im wesentlichen komplementär zu einem Bereich der kodierenden Region der mRNA ist, die für das Zielprotein kodiert, und das Formulieren desselben zu einem therapeutischen Agens umfaßt.

2. Verfahren zum Herstellen eines therapeutischen Agens nach Anspruch 1, **dadurch gekennzeichnet**, daß das Oligonukleotid in Form eines Phosphotriesters hergestellt wird.

FIG.1

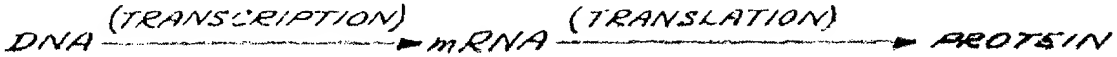

FIG.2

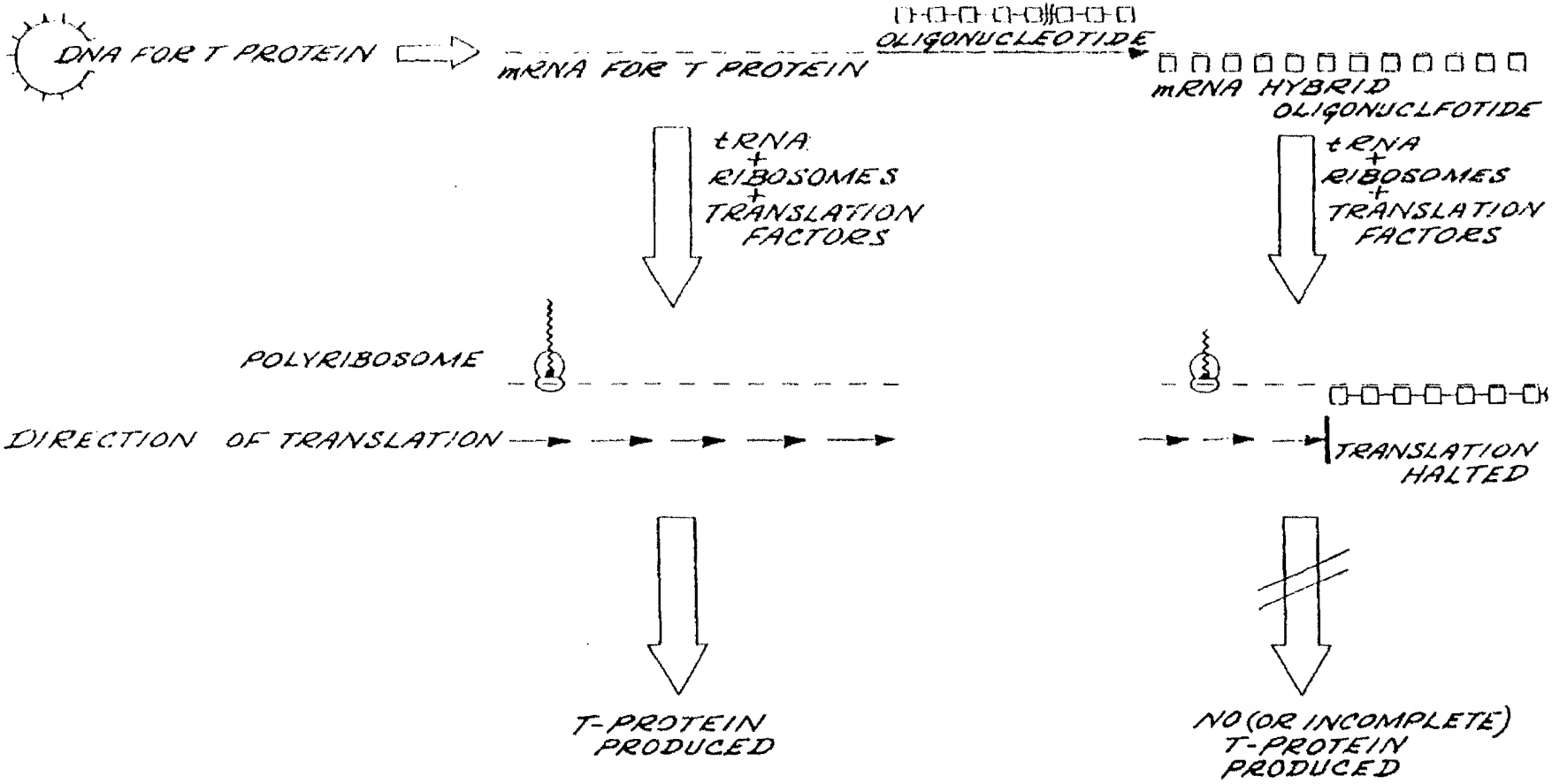

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FIG. 3

OLIGONUCLEOTIDE SPECIFIC FOR SV-40 T PROTEIN

VIRAL DNA(CODING) 3'...GAAACGTTTCTACCTATTTC...5'

VIRAL T PROTEIN mRNA 5'...CUUUGCAAAGAUGGAUAAAG...3'

T PROTEIN SPECIFIC OLIGONUCLEOTIDE 3'...GAAACGTTTCTACCTATTTC...5'

FIG. 7

OLIGONUCLEOTIDE SPECIFIC FOR FOLLICLE STIMULATING HORMONE

PROTEIN SEQUENCE:
N TERMINUS....THR TRP CYS ALA GLY TYR CYS TYR THR...C TERMINUS

mRNA PREDICTED:
5'END ACX UGG UGC GCX GGX UAC UGU UAC ACX 3'END

FSH OLIGONUCLEOTIDE FAMILY:
3'END TCG(T) ACC ACC CGG(T) CCG(T) ATG ACG ATG TG- 5'END

FIG. 4

CONSTRUCTION OF HIGH YIELD PLASMID CONTAINIING THE T PROTEIN OLIGONUCLEOTIDE

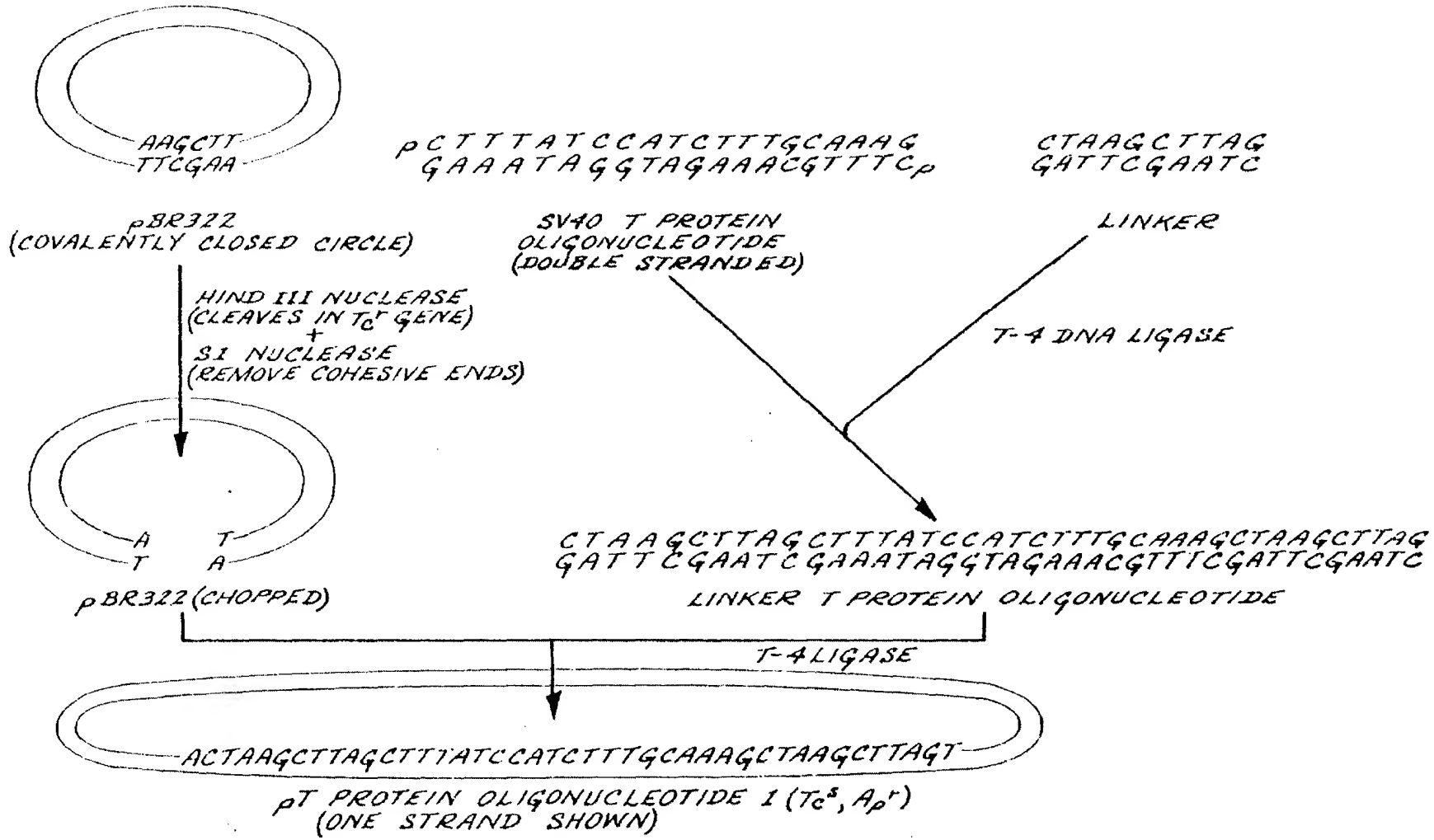

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FIG. 5

PRODUCTION OF SV-40 T PROTEIN OLIGONUCLEOTIDE FROM pT OLIGONUCLEOTIDE USING RESTRICTION NUCLEASE CLEAVAGE TO RELEASE FRAGMENTS.

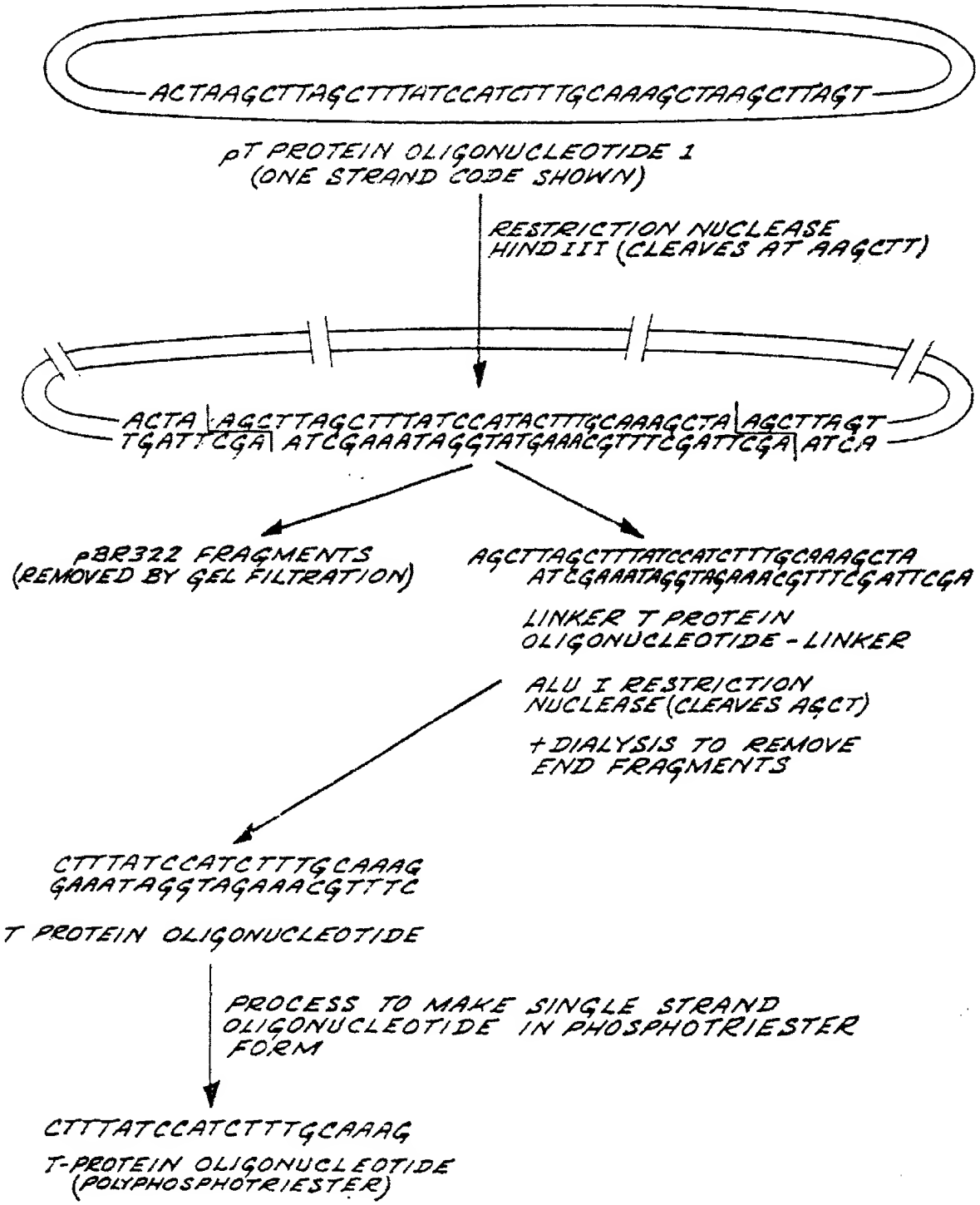
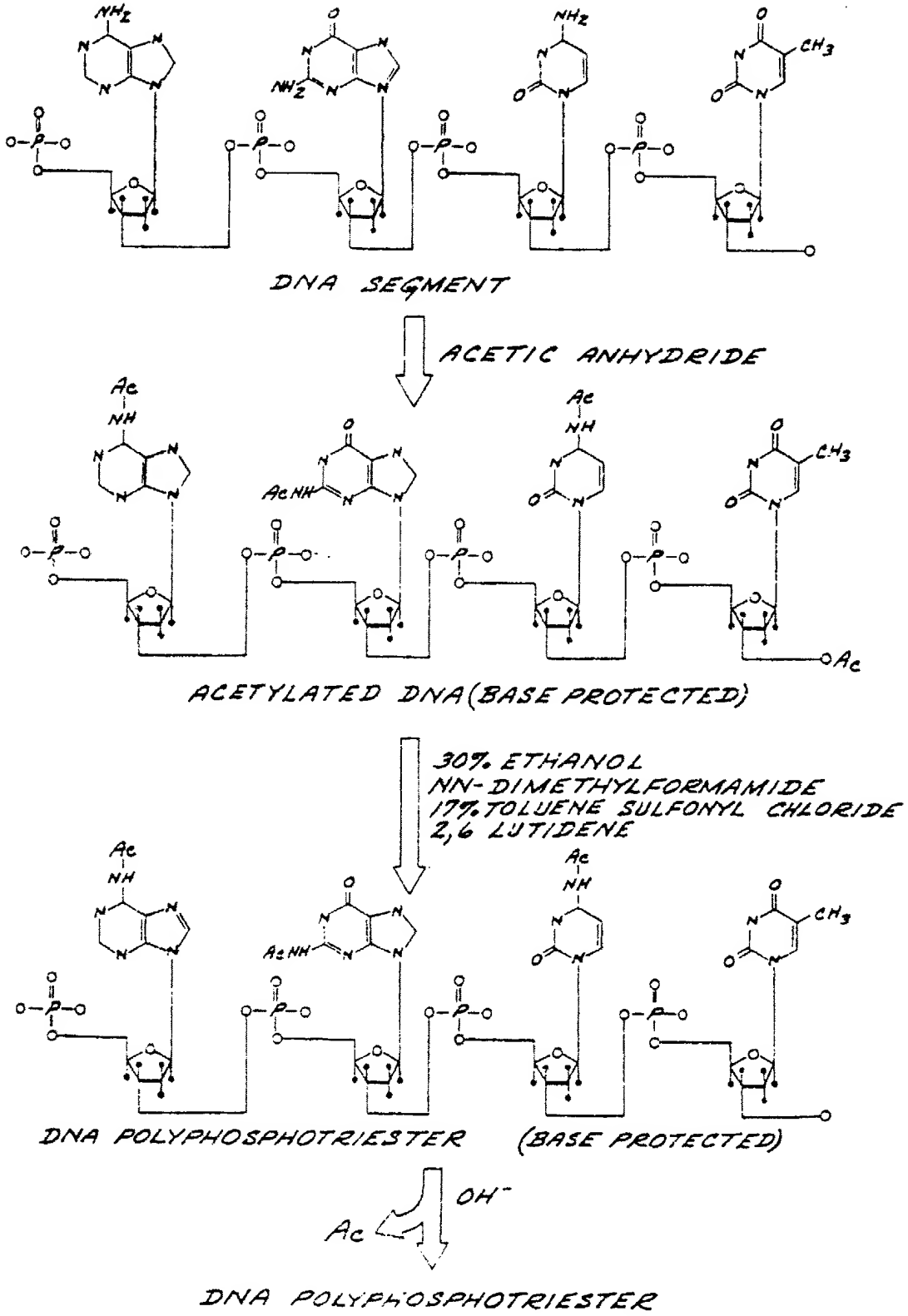


FIG.6


DNA SEGMENT
ACETIC ANHYDRIDE
ACETYLATED DNA (BASE PROTECTED)
30% ETHANOL
NN-DIMETHYLFORMAMIDE
17% TOLUENE SULFONYL CHLORIDE
2,6 LUTIDENE
DNA POLYPHOSPHOTRIESTER
(BASE PROTECTED)
OH⁻
DNA POLYPHOSPHOTRIESTER